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Phenotype Array Analysis of Metabolic Differences in Yersinia pestis

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Phenotype Array Analysis of Metabolic Differences in *Yersinia pestis*

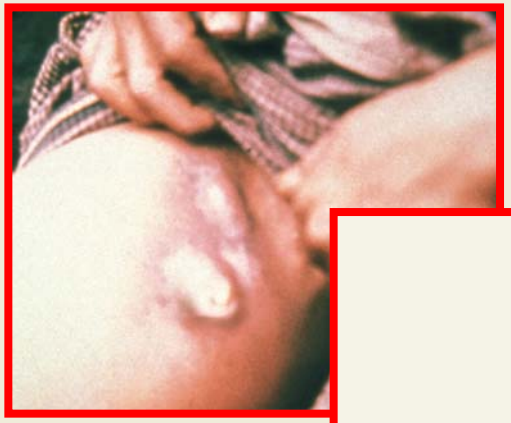
Ann E. Holtz-Morris

Lawrence Livermore National Laboratory

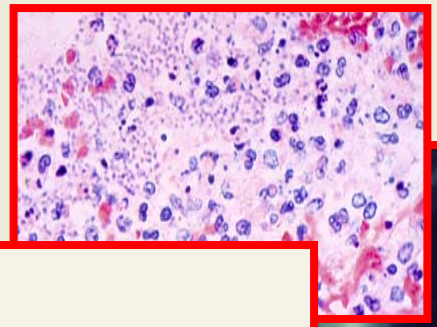
Outline

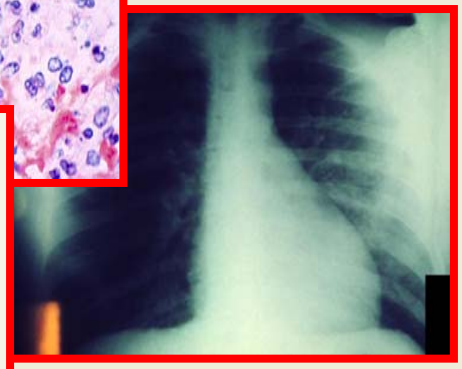
I. Introduction and background
II. Phenotype array technology
III. Results
IV. Analyzing data
V. Linking to virulence
VI. Future

Plague

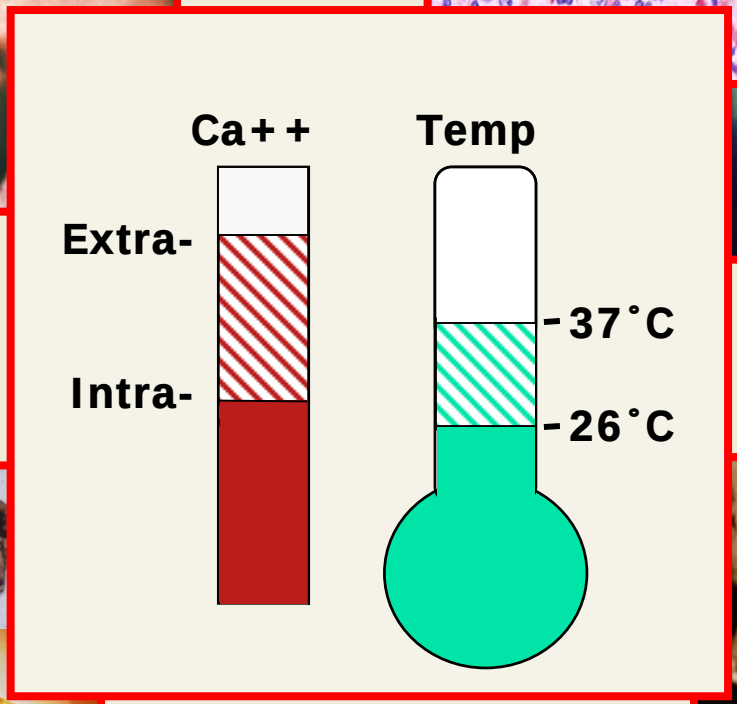


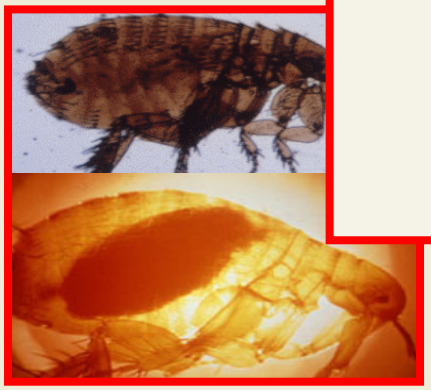
Bubonic





Pneumonic



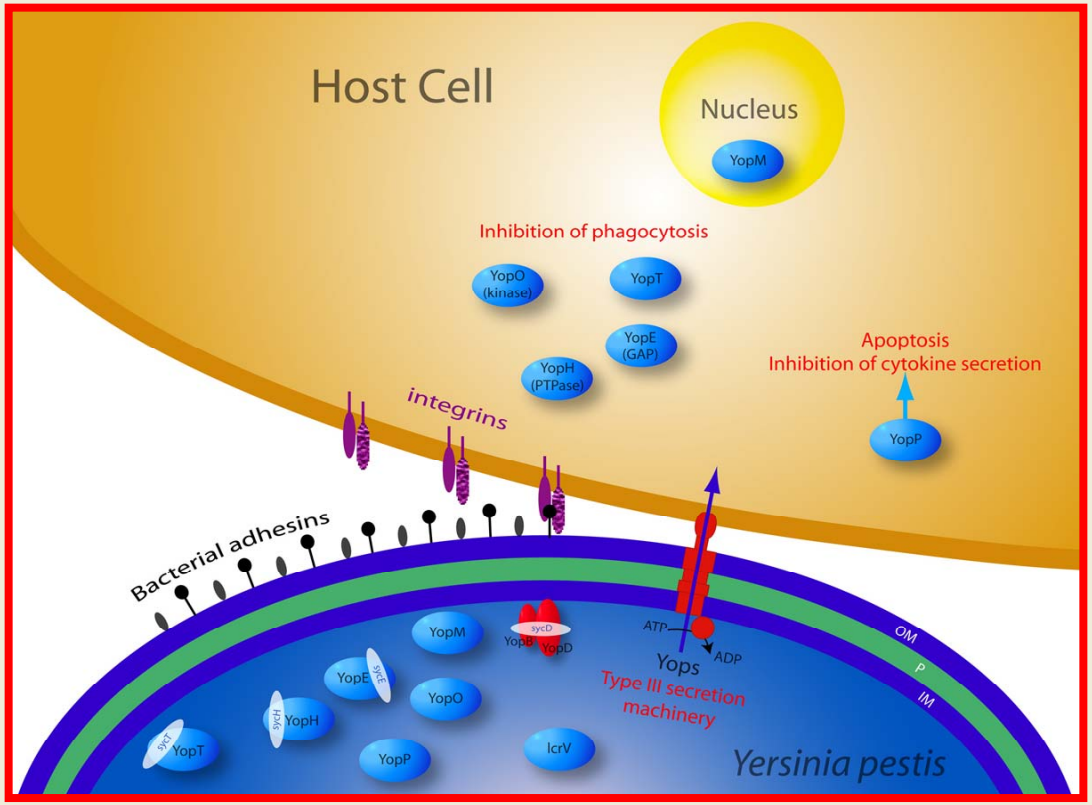


Flea

Rodent

http://phil.cdc.gov

Host-pathogen Interactions

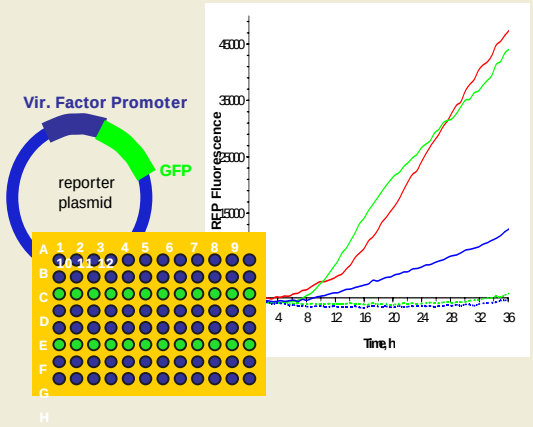


- Low Calcium Response
  - Intracellular environment
  - Induces TTSS
  - Bacteriostasis

- **Signatures for Early Detection of Exposure, Pathogen ID, Emerging and Engineered Threats, Antibiotic Resistance, Lethality, Dose**
- **Prevention, Detection, Containment, Treatment, Attribution**
- **Characterize pathogen, environmental and host factors that influence virulence**

Pathogen Characterization

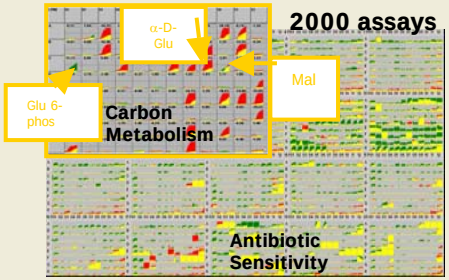
Real-time Expression



Virulence factor Real-time expression

Fluorescent reporter

Phenotype Arrays

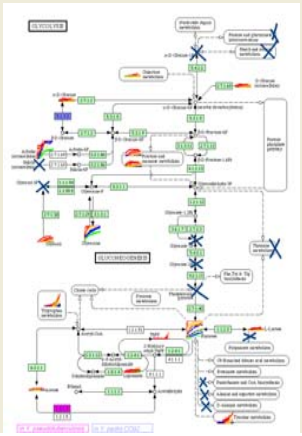


2000 assays

***Y. pestis* function**

Phenotype differences

- **Between growth conditions**
- **Between diverse strains**

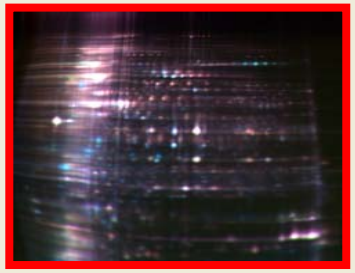


- Biochemical Pathways
- Systems Biology

Characterize pathogen, environmental and host factors that influence virulence

Proteomics

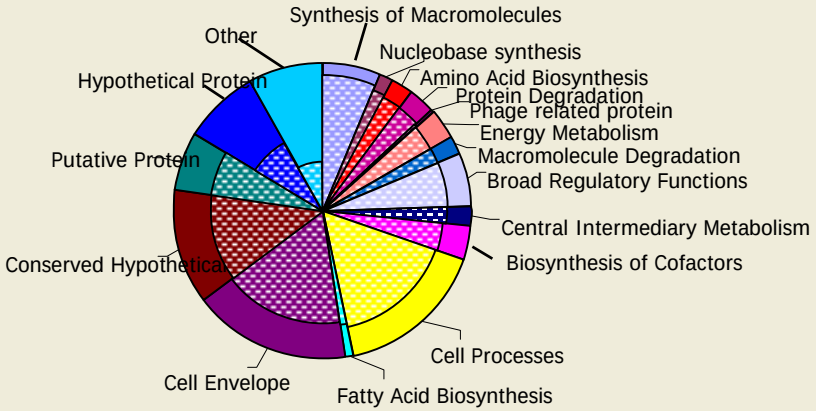
2D-DIGE



***Y. pestis* proteome**
26°C high calcium (flea)
37°C low calcium (human)
>2800 proteins spots
239 differential
ID by mass spec

•Proteomic diversity in *Y. pestis* strains and environmental conditions

Mass Spec

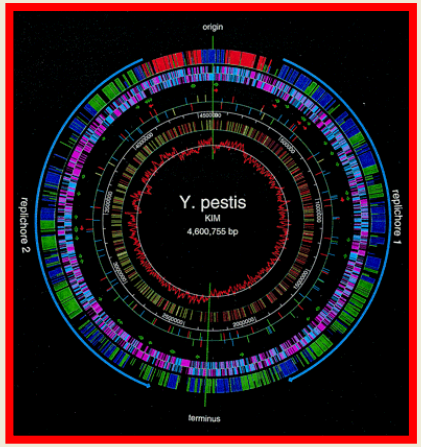


Collaboration with Drs. M. Lipton, D. Smith
>5000 Proteins from annotation, ~35% coverage

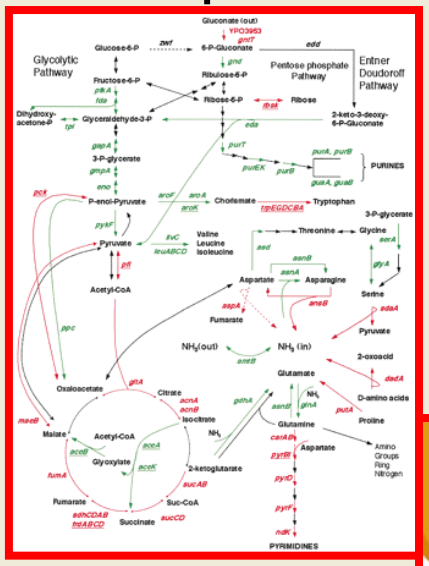
Holtz et al. 2004 *ASCB*
Forde et al. 2004 *BBRC*
Chromy et al. 2005 *J Bact*
Hixon et al. (submitted)
Forde et al. 2004 *BBRC*
Clatworthy et al. in prep

Systems Biology of *Y. pestis*

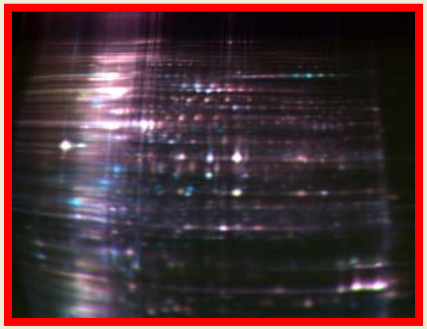
Genomics[1,2,3]



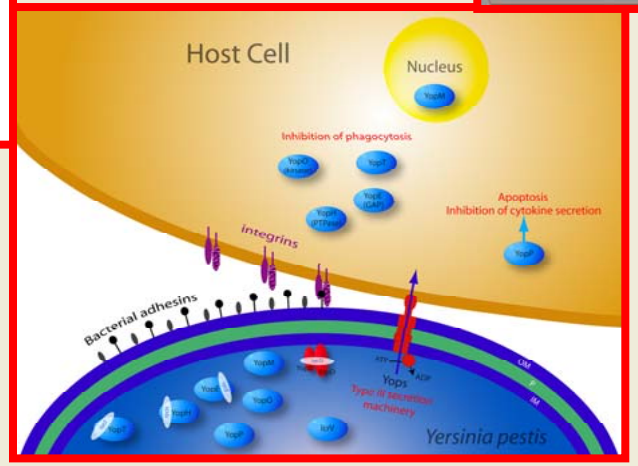
Transcriptomics[4]



Proteomics[5]

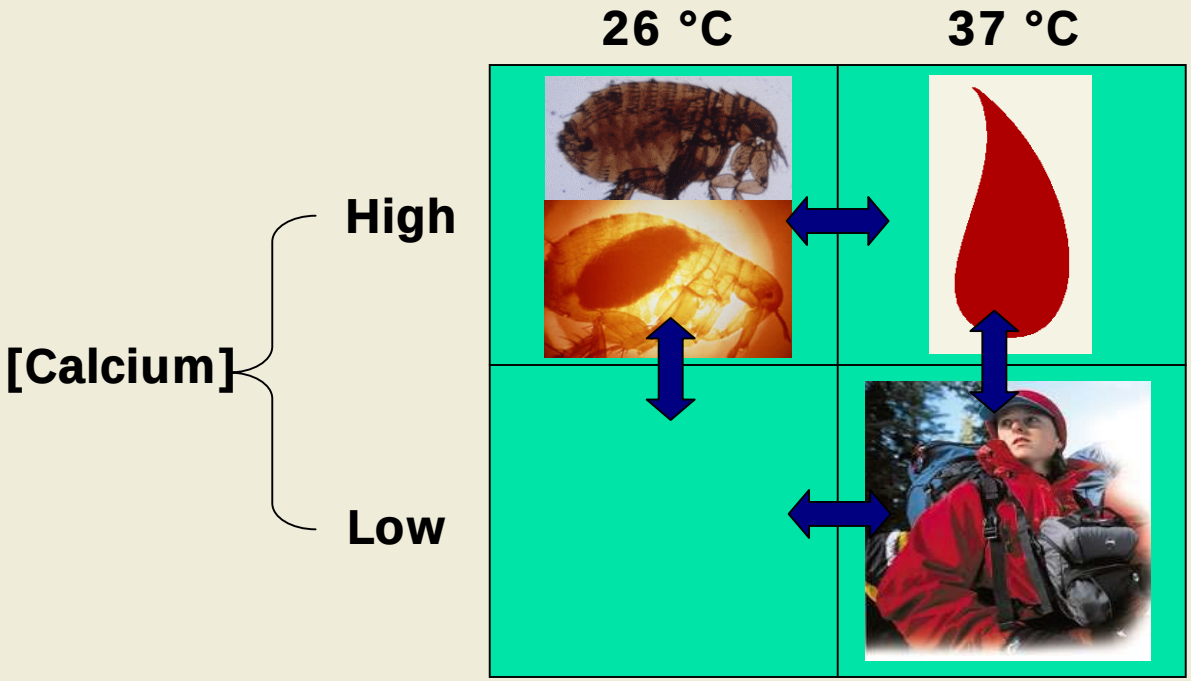


Functionomics[6]



[1] Parkhill *et al.* 2001 Nature
[2] Deng *et al.* 2002. J Bacteriol
[3] Song *et al*. 2004 DNA Res.
[4] Motin *et al.* 2004. J Bacteriol
[5] Chromy *et al.* 2005. J Bacteriol
[6] Holtz-Morris et al. in preparation

Y. pestis Physiological Growth Conditions (Flea Vs. Human)

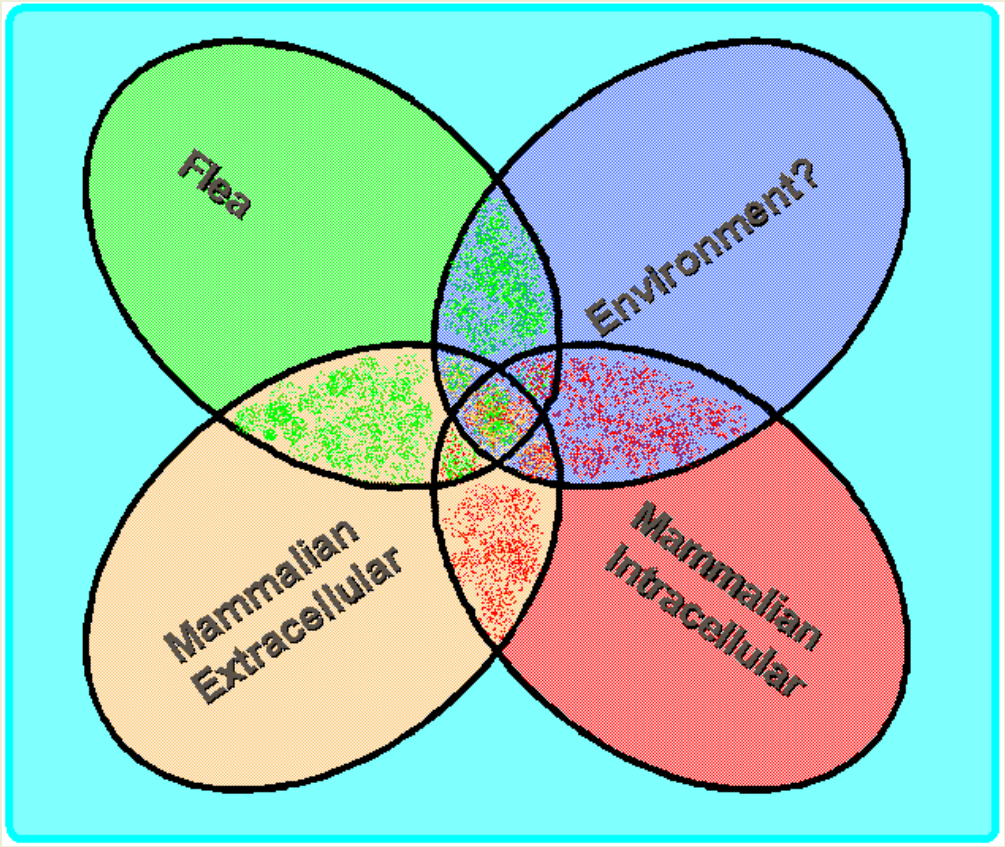


Basic Metabolism

- Original papers (1940s to 1960s) did not account for differences due to low calcium response and subsequent TTSS.
- It is now known that significant differences in mRNA expression[1] and protein expression[2] occur between flea vector, mammalian extracellular, and mammalian intracellular environments.

[1]Motin et al. 2004 J Bacteriol
[2]Chromy et al. 2005 J Bacteriol

Expected Metabolic Activity



Hypothesis: Different metabolic pathways may be used in different environments: unique, union or intersection.

Applications of Results

- Pathogen characterization to support detection and forensics
  - Differential - flea vs. human physiological conditions
  - Strain diversity – typing and attributes
    - Eg. 2004-2005 Congo outbreak, 57dead/130cases – why pneumonic[1,2]?
    - Eg. Possible engineered pathogens
- Biomarkers and virulence factors
  - What influences their expression?
  - Can a false negative, *i.e.* the pathogen is present but the detection method fails because the marker is missing, occur due to changes caused by the growth conditions, as in case of F1 antigen?[3]
  - Identify virulence pathways and tie to host response in next generation arrays
- Contribute to systems biology view of virulence

[1]http://www.cidrap.umn.edu/cidrap/content/bt/plague/news/mar1505plague.html

[2]http://www.cidrap.umn.edu/cidrap/content/bt/plague/news/feb1805plague.html

[3]Mahesh *et al*. 2005 Comp Immunol Microbiol Infect Dis

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Phenotype Arrays

Omnilog robot

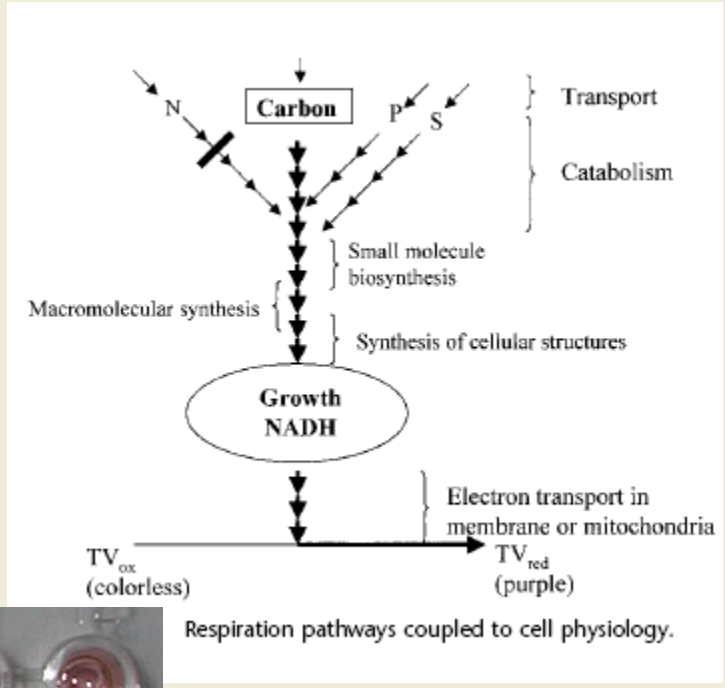
PM1: Carbon sources

PM 1-	01	02	03	04	05	06	07	08	09	10	11	12
A	Negative Control	L-Arabinose	N-Acetyl-D-Glucosamine	D-Saccharic Acid	Succinic Acid	D-Galactose	L-Aspartic Acid	L-Proline	D-Alanine	D-Trehalose	D-Mannose	Dulcitol
B	D-Serine	D-Sorbitol	Glycerol	L-Fucose	D-Glucuronic Acid	D-Gluconic Acid	D,L-α-Glycerol-Phosphate	D-Xylose	D,L-Lactic Acid	Formic Acid	D-Mannitol	L-Glutamic Acid
C	D-Glucose-6-Phosphate	D-Galactonic Acid-γ-Lactone	D,L-Malic Acid	D-Ribose	Tween 20	L-Rhamnose	D-Fructose	Acetic Acid	α-D-Glucose	Maltose	D-Melibiose	Thymidine
D	L-Asparagine	D-Aspartic Acid	D-Glucosaminic Acid	1,2-Propanediol	Tween 40	α-Keto-Glutaric Acid	α-Keto-Butyric Acid	α-Methyl-D-Galactoside	α-D-Lactose	Lactulose	Sucrose	Uridine
E	L-Glutamine	M-Tartaric Acid	D-Glucose-1-Phosphate	D-Fructose-6-Phosphate	Tween 80	α-Hydroxy Glutaric Acid-γ-Lactone	α-Hydroxy Butyric Acid	β-Methyl-D-Glucoside	Adonitol	Maltotriose	2-Deoxy Adenosine	Adenosine
F	Glycyl-L-Aspartic Acid	Citric Acid	M-Inositol	D-Threonine	Fumaric Acid	Bromo Succinic Acid	Propionic Acid	Mucic Acid	Glycolic Acid	Glyoxylic Acid	D-Cellobiose	Inosine
G	Glycyl-L-Glutamic Acid	Tricarballylic Acid	L-Serine	L-Threonine	L-Alanine	L-Alanyl-Glycine	Acetoacetic Acid	N-Acetyl-β-D-Mannosamine	Mono Methyl Succinate	Methyl Pyruvate	D-Malic Acid	L-Malic Acid
H	Glycyl-L-Proline	p-Hydroxy Phenyl Acetic Acid	m-Hydroxy Phenyl Acetic Acid	Tyramine	D-Psicose	L-Lyxose	Glucuronamide	Pyruvic Acid	L-Galactonic Acid-γ-Lactone	D-Galacturonic Acid	Phenylethyl-amine	2-Amino ethanol

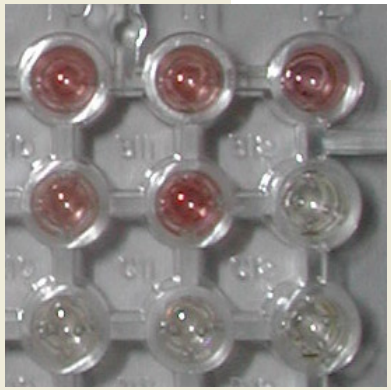
PM plates

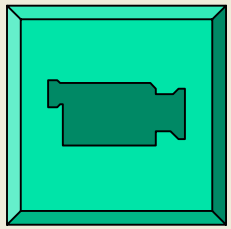
	+Ca	-Ca
26°C Flea	Green	Blue (control)
37°C Mammalian	Gold Extracellular	Red Intracellular

Phenotype Array Technology



Respiration pathways coupled to cell physiology.





- Measures dye produced by coupled redox reaction to respiration
- Change in intensity indicates metabolism of the substrate in that well
- Inoculum is in gelling media IF-0 or IF-10
- Optics system converts color into a dye intensity value

Bochner 2003 Nat Rev Genet

Bochner *et al.* 2001 Genome Res

High Throughput Screening: 2000 Chemicals Simultaneously

- Minimal medium
  - Carbon sources
  - Nitrogens
  - Phosphates
  - Sulfurs
  - Amino acids
  - Dipeptides
  - Tripeptides
- Rich medium
  - Osmotic stressors
  - pH effects
  - Antibiotics

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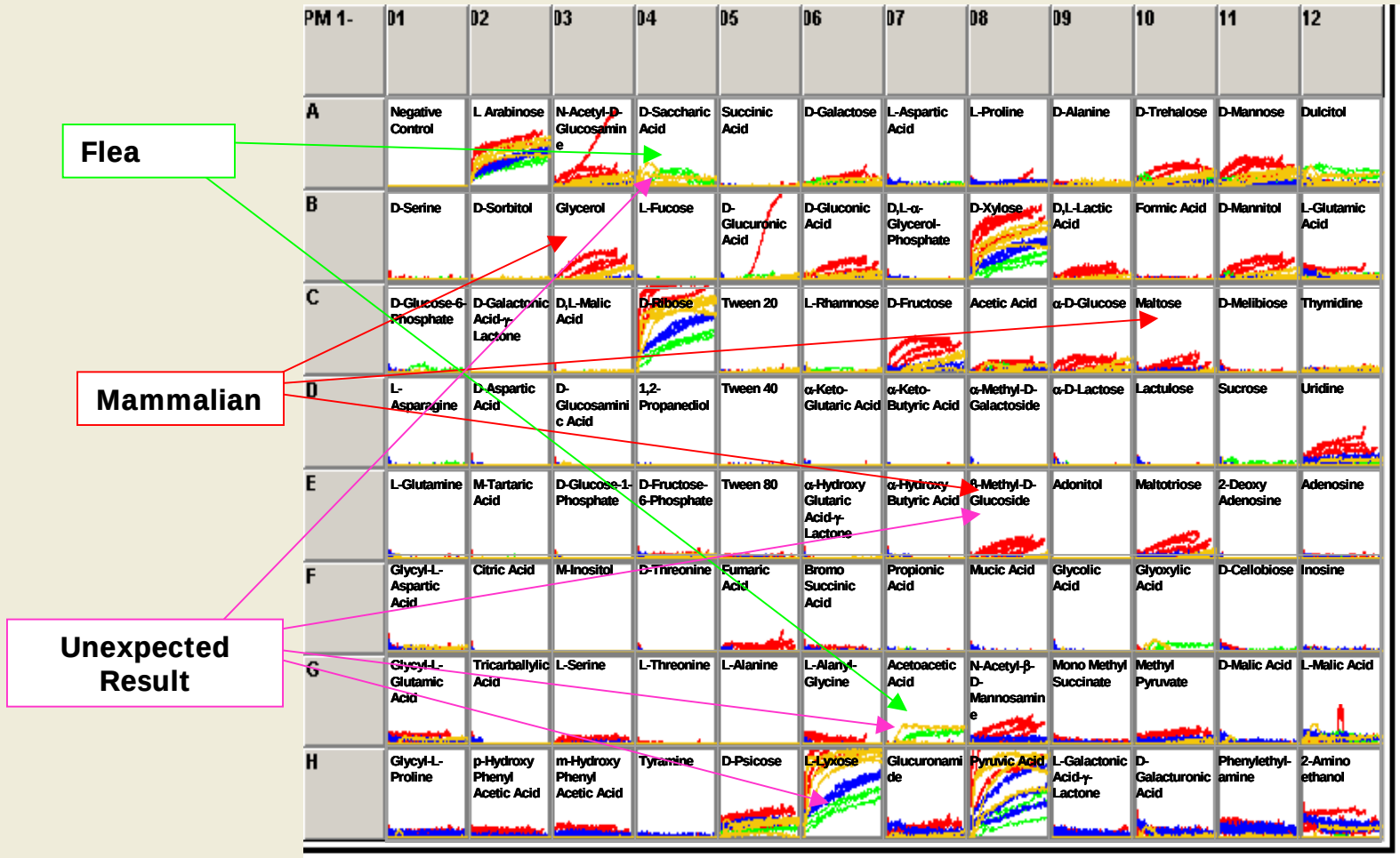
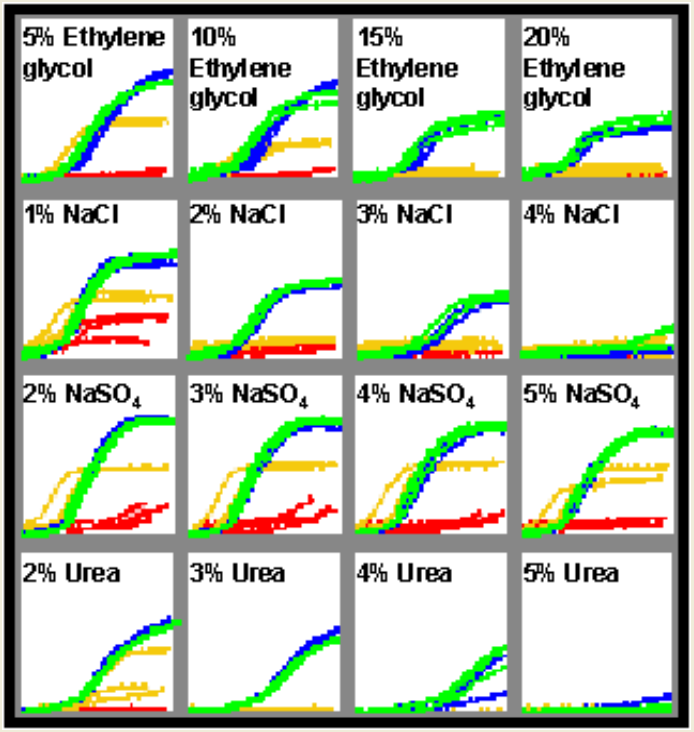
Functional Phenotype

	+Ca	-Ca
26°C Flea	Green	Blue (control)
37°C Mammalian	Gold Extracellular	Red Intracellular

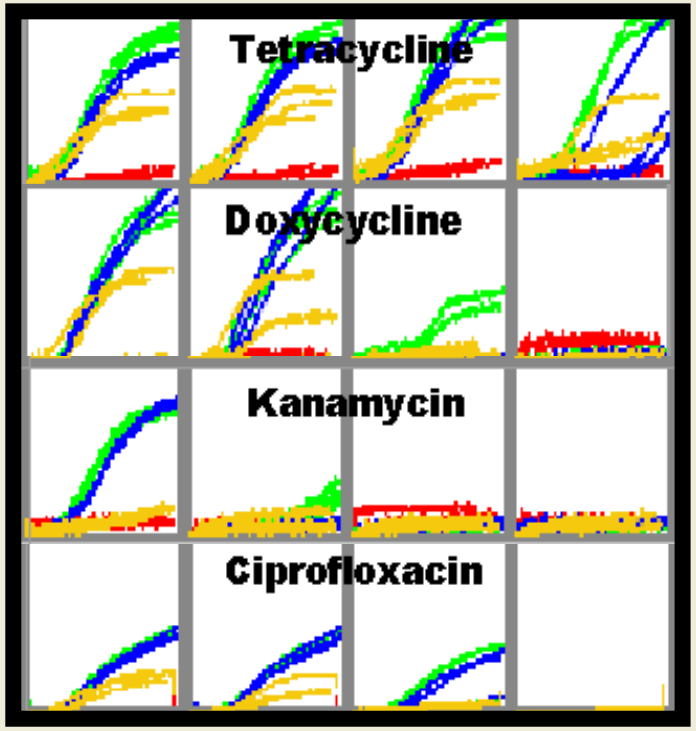


Osmotic Stressors and Antibiotics

Osmotic Stressors



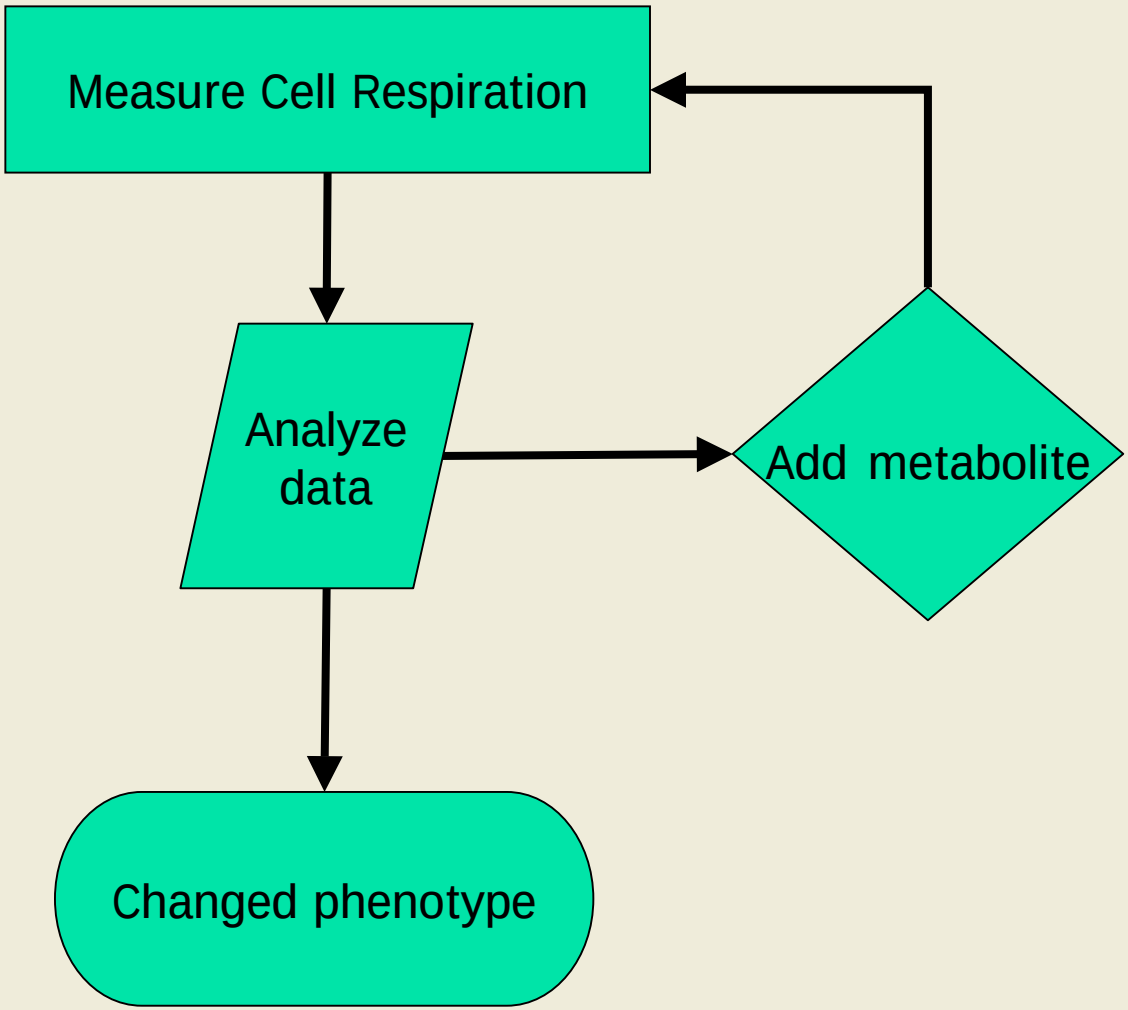
Drug concentration



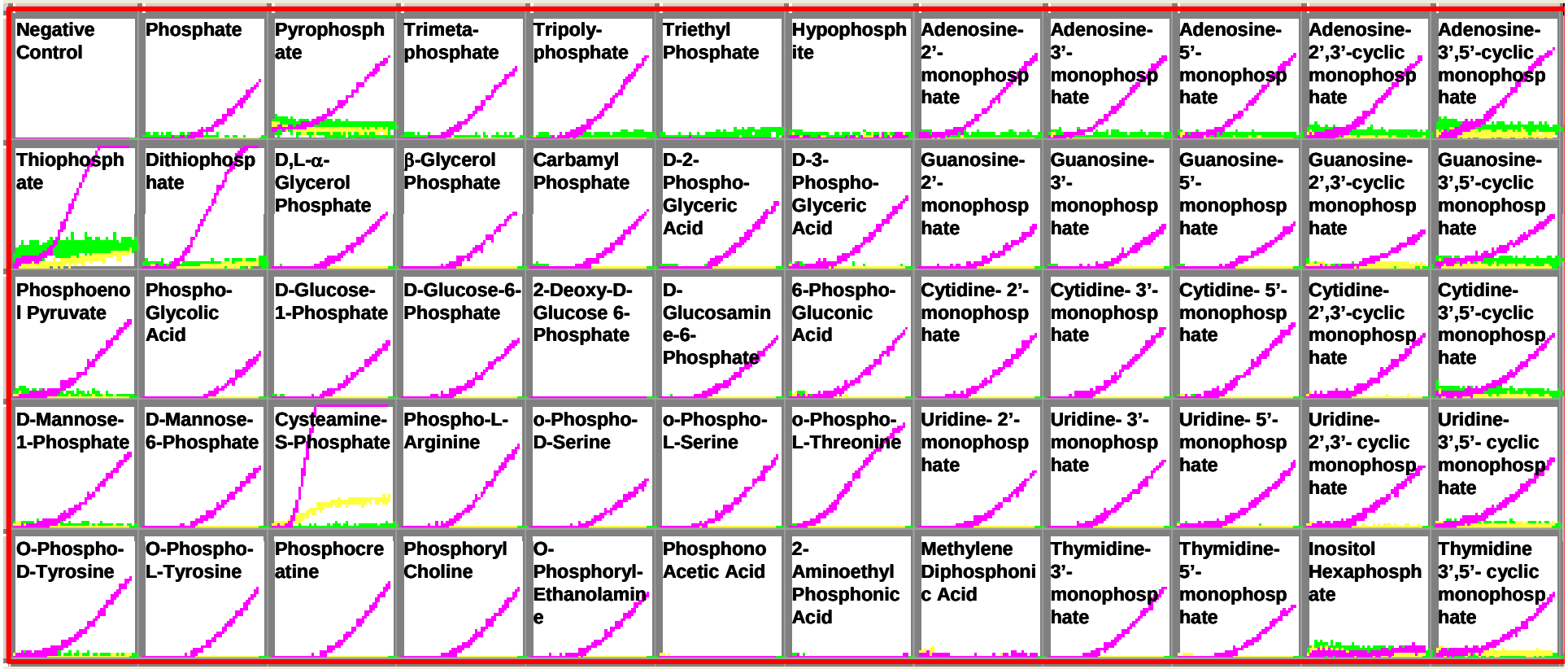
- Response to stressors and antibiotics varies with calcium and temperature.

	+Ca	-Ca
26°C Flea	Green	Blue (control)
37°C Mammalian	Gold Extracellular	Red Intracellular

Flow Chart of Reiteration



Supplement Changes Phenotype



Flea: 26°C +Ca
+Met
+Cys

Chemical Bins

- Growth conditions
  - Compounds used at 37°C
    - Only at low calcium
    - Only at high calcium
    - Irrespective of calcium
  - Compounds used only at 26°C, irrespective of calcium
  - Compounds used at both 37°C and 26°C
    - Some better at 37°C
    - Some equally well at 26°C
  - Compounds used only in presence of calcium, regardless of temperature
  - Compounds not used

Chemical bins (cont'd)

- Possible inhibitory, Val-X
- Compounds used only when methionine or cysteine or tryptophan supplemented
  - *eg.* Tween 20, 40, and 80 +Cys
  - *eg.* Phosphorylated substrates at 26°C +Ca +Met

Comparing Strains:

- Y. pestis KIM D27: pgm-, conditionally virulent
- Y. pestis NYC: virulent, recent bubonic clinical isolate
  - is 1000X more lethal than India 195/p in mouse model
- Y. pestis India 195/p: virulent, pneumonic clinical isolate

Strain Dependent Phenotype

- Most strains used compounds similarly
- Methionine precursors used only by KIM D27 and only at 26°C

Respiration at 37°C+Ca Vs. 26°C-Ca

chemical	India 195/p	NYC	KIM D27
glycerol	-	-	37°C {+} 26°C {-}
N-Acetyl-Neuraminic Acid	37°C {+} 26°C {-}	37°C {+} 26°C {-}	
Methionine and precursors	-	-	37°C {-} 26°C {+}
pH5.5	37°C {-} 26°C {+}	-	-

The patterns of chemical utilization can differentiate strains.

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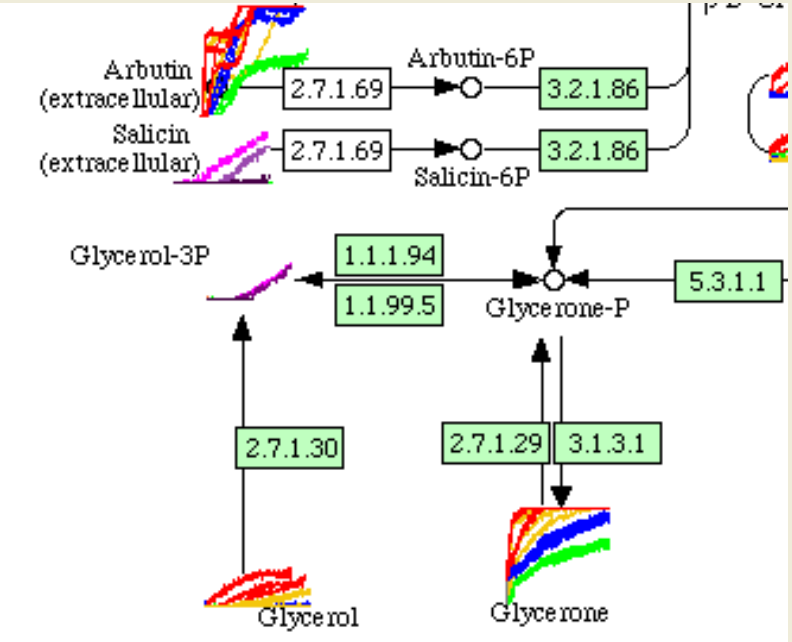
The Data Explodes

- Studies are reiterative
- Software is necessary
- 4 hours “hands on” to 40 hour data analysis

Analyzing Data: Use of KEGG[1] Maps

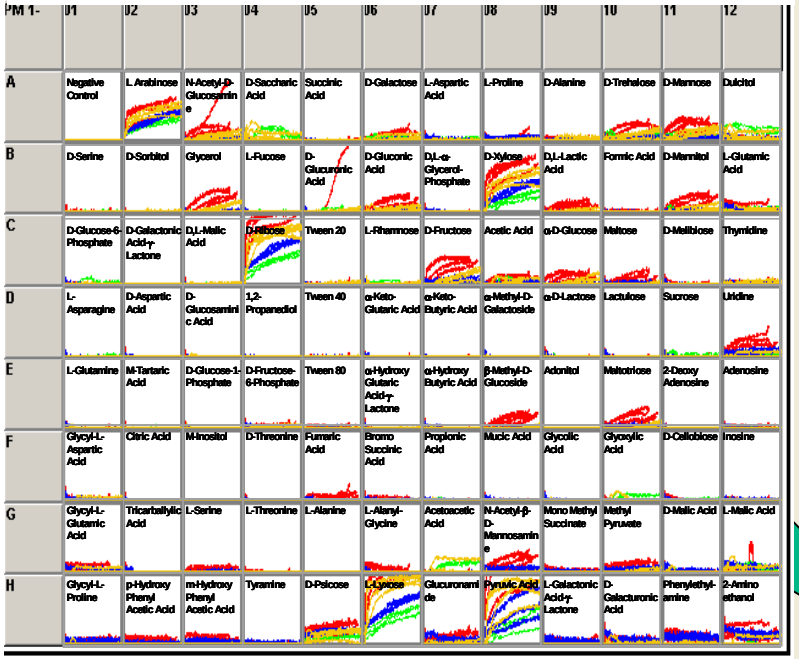


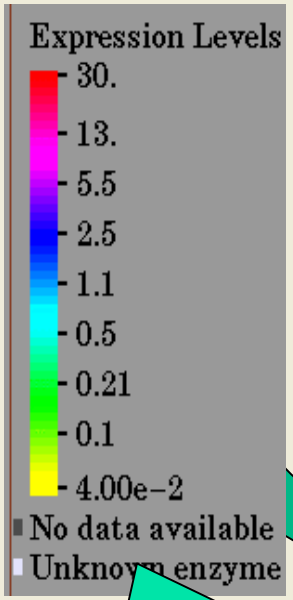
- Points out unannotated metabolic activities
- Points out activities not expressed under the conditions

[1]Kanehisa *et al.* 2004 Nucleic Acids Res

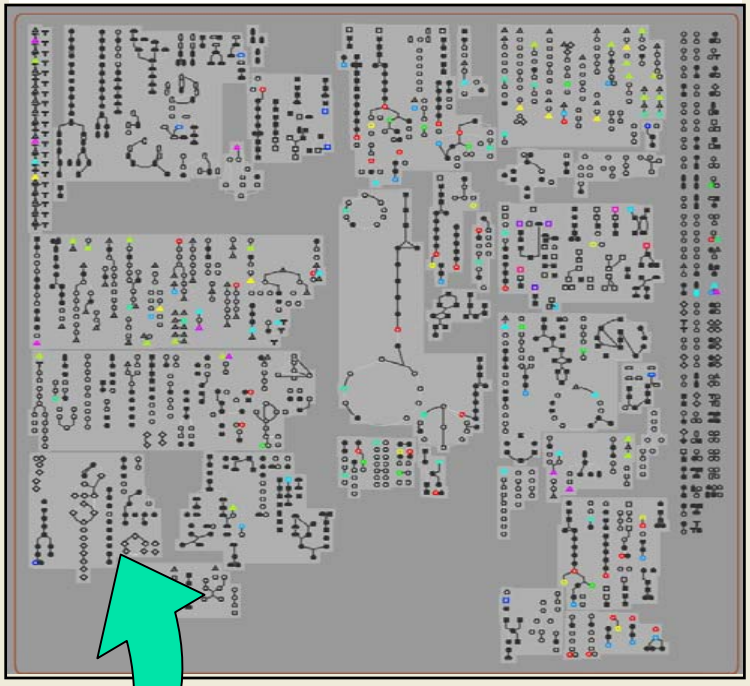
Visualizing Phenotype Data

Functional phenotype

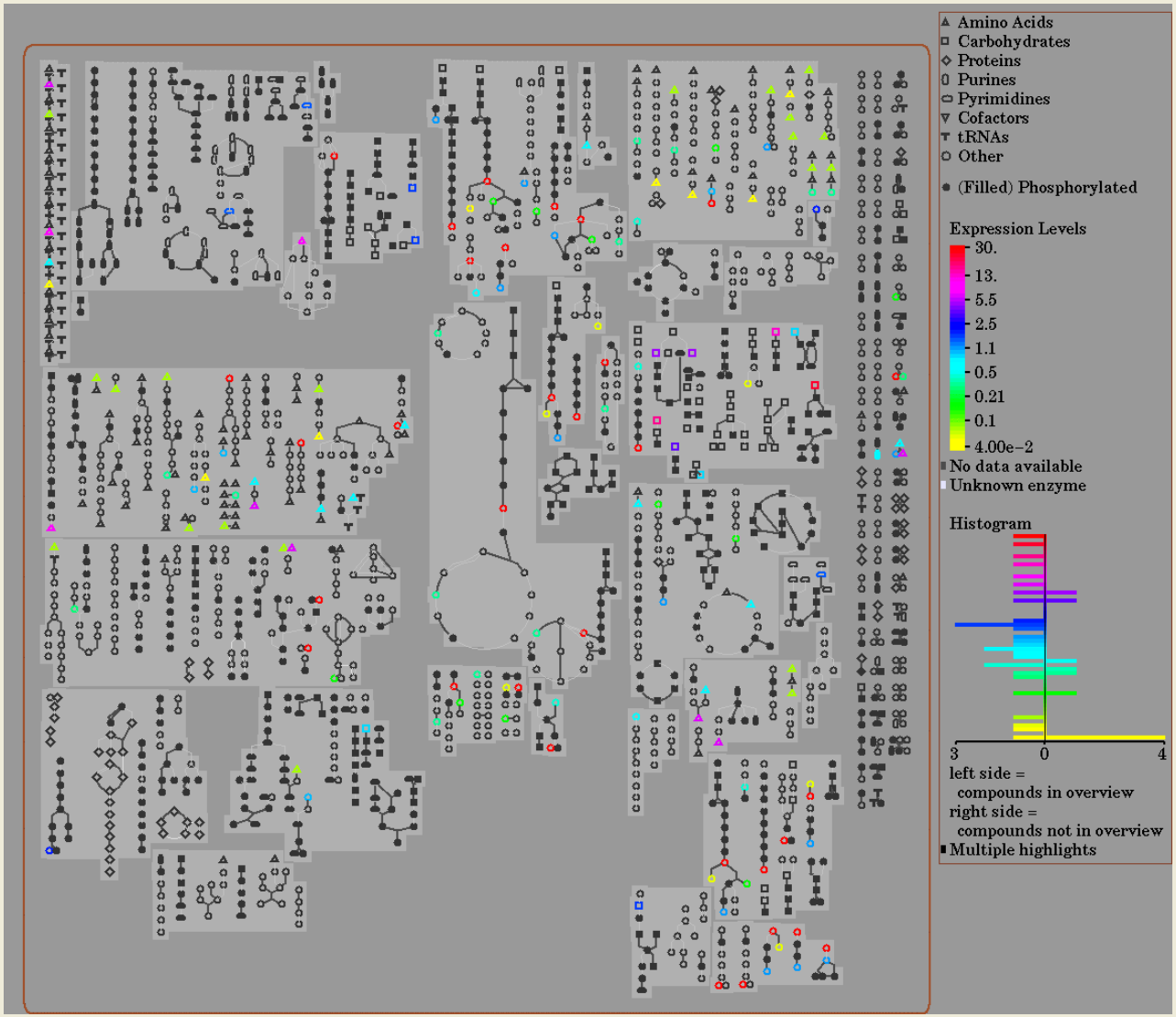




Metabolic overview map



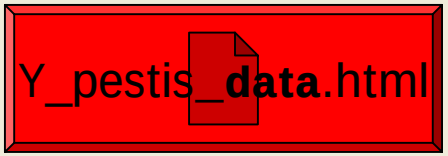
Use of Biocyc Map



- Comparing changing conditions
- Examining global relationships
- Alternative pathways

Y pestis KIM PGDB courtesy Peter Karp, SRI International

Y. pestis KIM Overview Maps



- *Y. pestis* KIM overview of 4 conditions
- Order
  - 26° +Ca
  - 26° -Ca
  - 37° +Ca
  - 37° -Ca
- Mass-spec protein data and substrate rate data combined

From Function to Gene...

- Nature Reviews Microbiology 3, 272 (2005)

  "When a genome has been completely annotated, there remain almost 40% of genes — many of which are conserved among several different species — for which no function can be predicted....Plus, experimental validation of predicted functions has lagged far behind the speed of annotation. In fact, an inverse pyramid of information is present, in which annotations of huge numbers of sequenced genes are based on a relatively tiny number of functionally characterized genes."

- We need to greatly improve the systems biology of *Y. pestis* to provide new diagnostic and therapeutic targets.

From Phenotype to Gene

Substrate → Function → Enzyme → Sequence → Gene

1: y0975 possible NAGC-like transcriptional regulator [*Yersinia pestis KIM*]
GeneID: 1145922 Locus tag: y0975
Transcripts and products: RefSeq below

NC_004088

Genomic context:

Gene type: protein coding
RefSeq status: Provisional
Organism: *Yersinia pestis KIM* (strain: KIM)
Lineage: *Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Yersinia*

Bibliography: Gene References into Function (GeneRIF): **Submit** help

PubMed links

General protein information

Name: possible NAGC-like transcriptional regulator
Comment:
NP_668305: COG classification Description: COG1940 [K] Transcriptional regulator/sugar kinase
Group: Transcription
Category: Information processing

NP_668305: residues 1 to 299 of 304 are 68.89 pct identical to residues 47 to 345 of 348 from E. coli K12 : B0394; residues 1 to 299 of 304 are 69.56 pct identical to residues >gb|AAG54741.1|AE005218_6 (AE005218) yajF gene product [Escherichia coli O157:H7 EDL933]

NCBI Reference Sequences (RefSeq)

Product NP_668305 possible NAGC-like transcriptional regulator [Yersinia pestis KIM]
Conserved Domains (1) summary
pfam00480: ROK; ROK family
Location: 4 - 193 Blast Score: 343

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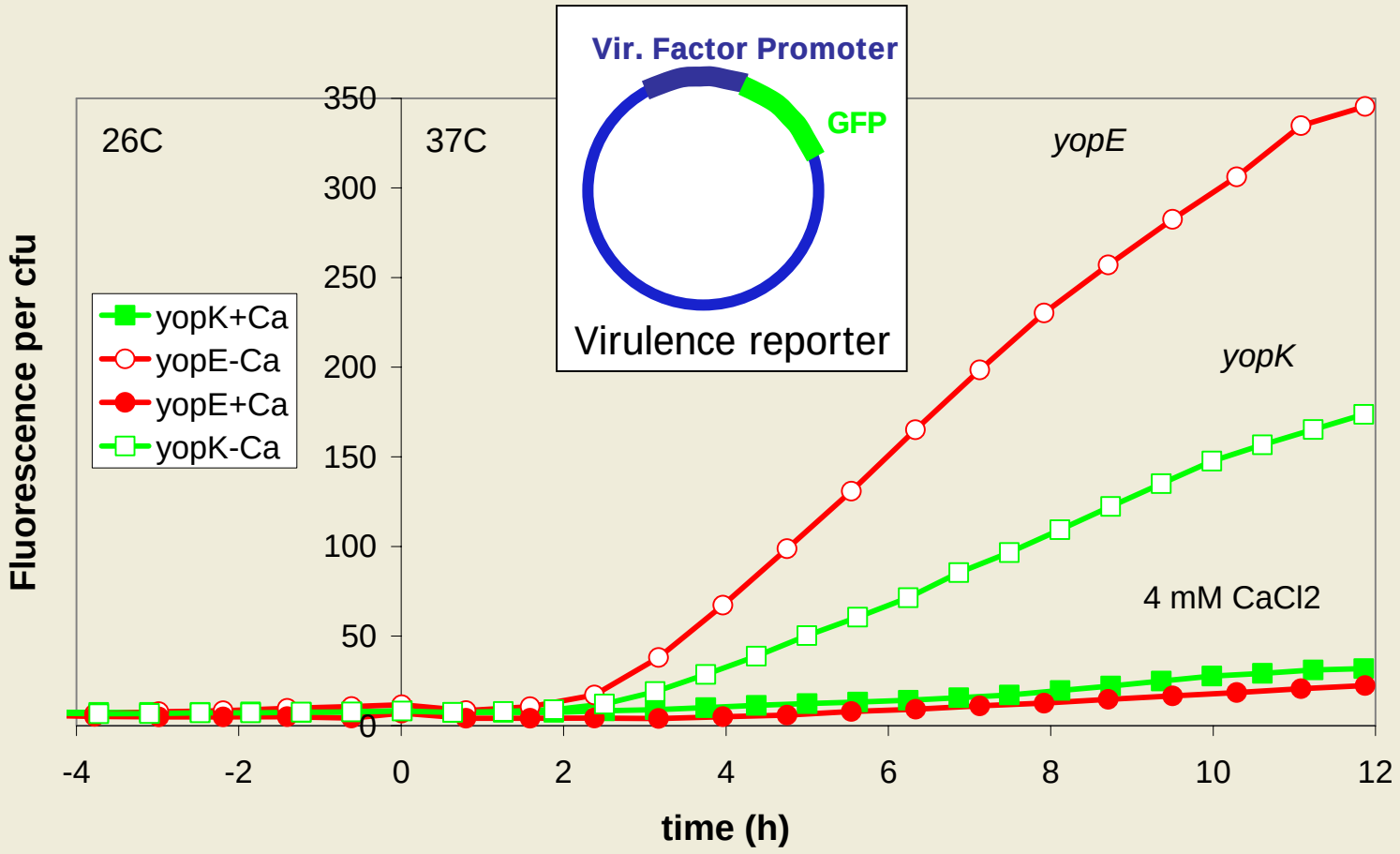
Real-time Characterization of *Y. pestis* Virulence Factors

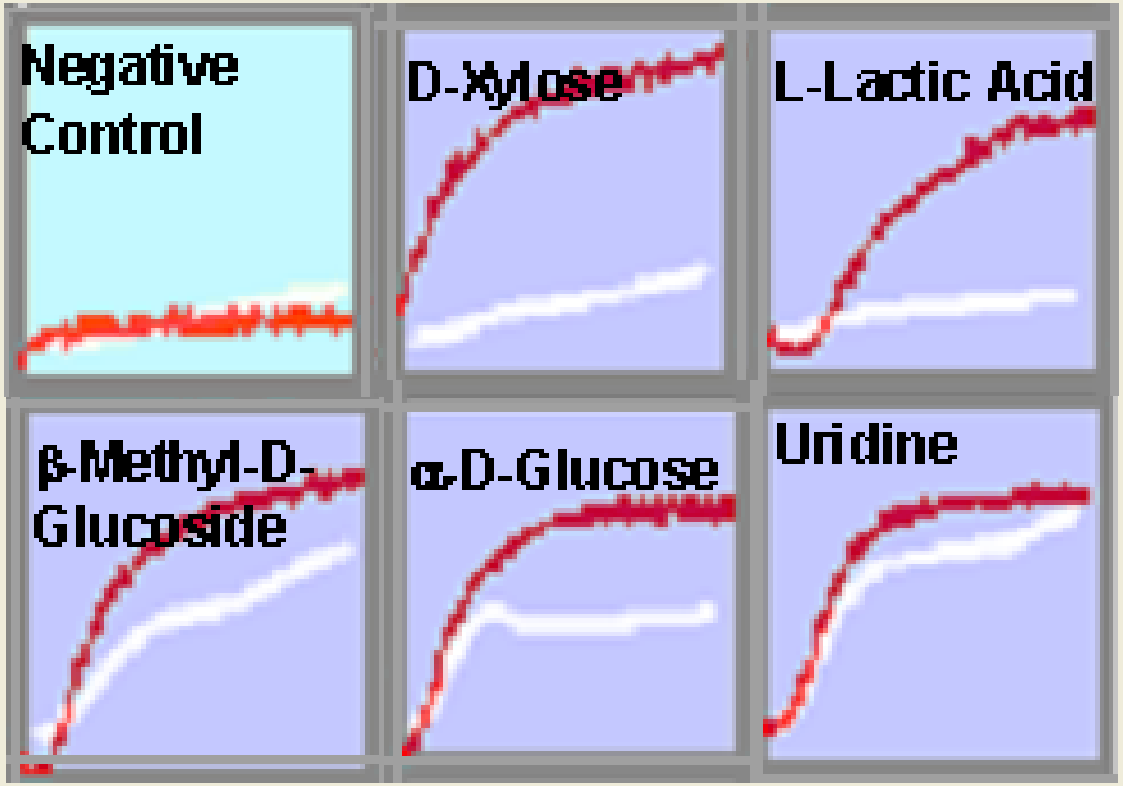


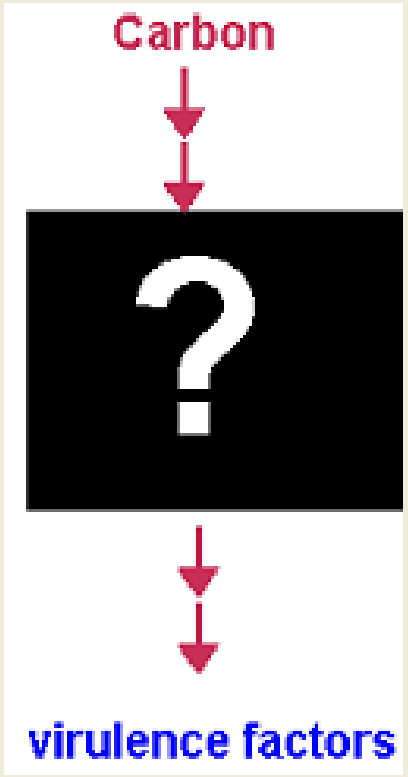
Measures activity of virulence factor promoters by fluorescent reporter system, estimating virulence factor expression

Forde et al. 2004 BBRC.

Linking YopE Expression to Phenotype

Functional phenotype changes virulence factor expression





Red is respiration; white is pYopE-GFP reporter

Outline

I. Introduction and background
II. Phenotype array technology
III. Results
IV. Analyzing data
V. Linking to virulence
VI. Future

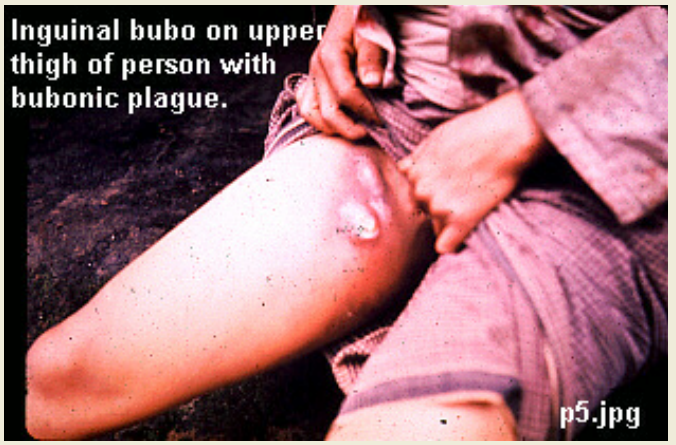
Next Generation Signatures/Biomarkers

- Qualitative information about a pathogen
  - Specific host response to pathogens
    - Differences between closely related organisms
  - Virulence factors of the pathogen
  - Antibiotic resistance, genetically engineered
- Panel of biomarkers needed to ID exposure
  - Naturally occurring and genetically engineered

Patient with Fever/Cough

Is it plague, anthrax, SARS, flu?

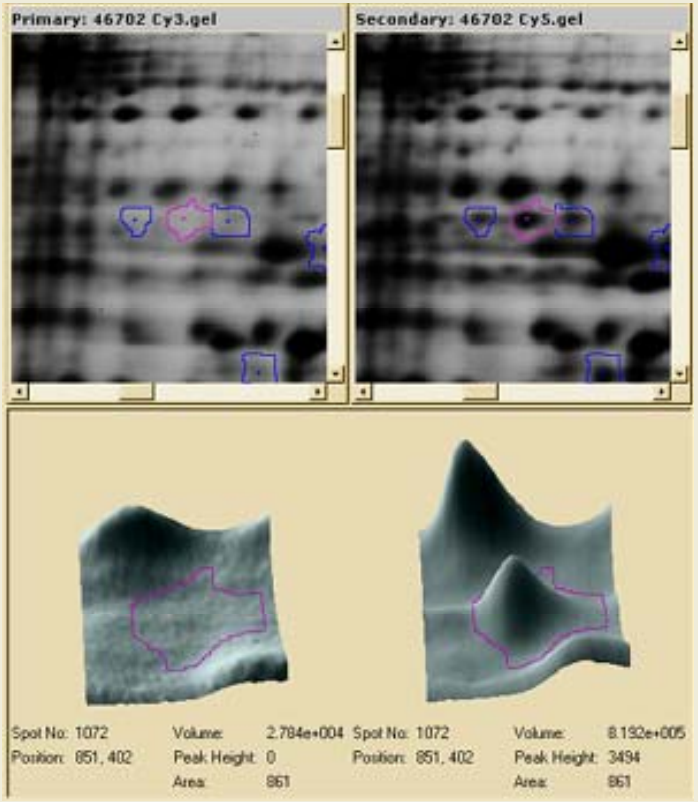
Do we have to wait for this ➔



Carbon Sources for Validating Biomarkers

Upcoming project

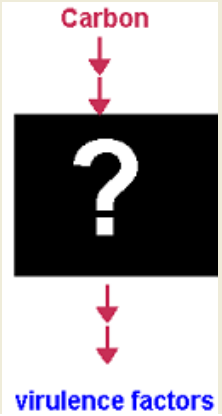
Carbon sources from Phenotype Arrays



No False Negatives!

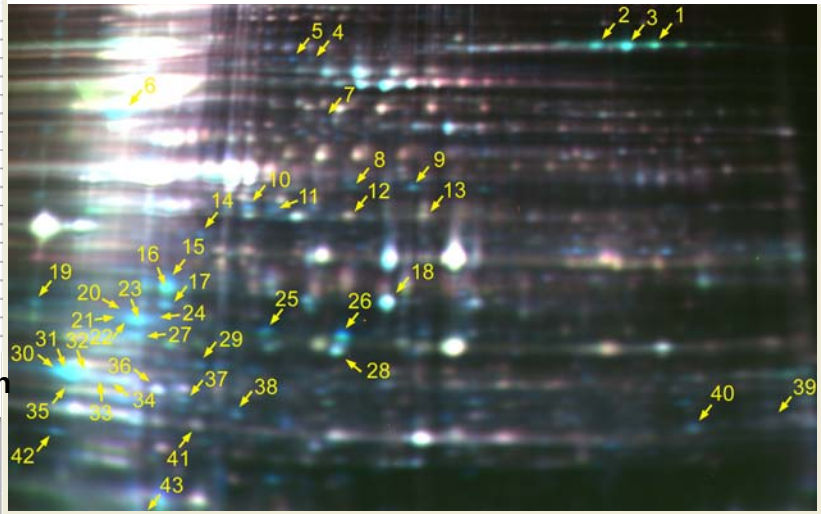
Data Integration and Future

Future: integrate metabolomics to verify biochemical pathways

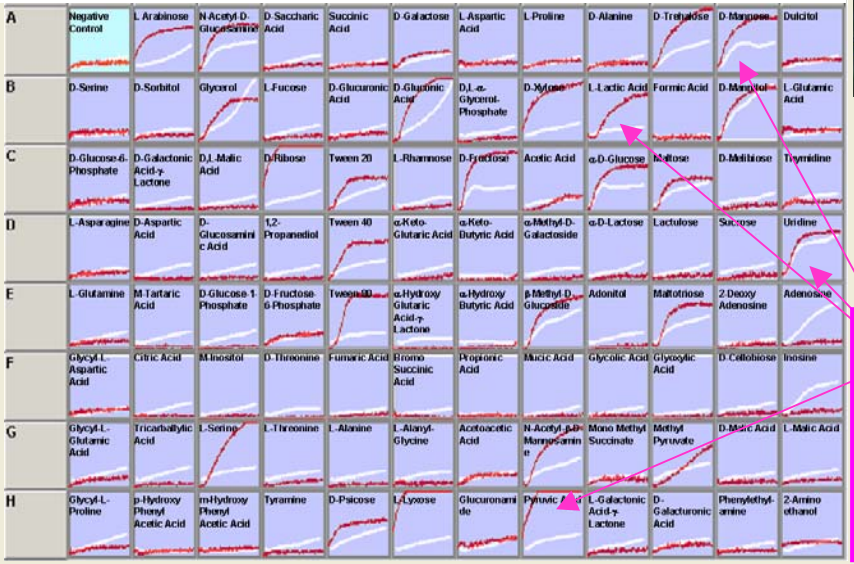


Spot Number(s)	Protein
	VIRULENCE-ASSOCIATED FACTORS
1-3, 8-9, 26, 38	catalase-peroxidase (KatY)
7, 29	murine toxin (Ymt)
18	Plasminogen activator (Pla)
19	F1 capsule antigen (Caf1)
	PUTATIVE VIRULENCE FACTORS
15	α-enolase (Eno)
25	outer membrane porin A (OmpA)
28	probable N-acetylmuramoyl-L-alanine amidase (AmpD3)
	METABOLIC ENZYMES
10	aspartate aminotransferase (AspC)
11b	phosphoglycerate kinase (Pgk)
12	maltose binding protein periplasmic protein precursor (MalE)
13	fructose-bisphosphate aldolase class II (Fba)
14	6-phosphogluconate dehydrogenase (Gnd)
16	D-3-phosphoglycerate (SerA)
39	trp repressor binding protein (WrbA)
40	glyceraldehyde 3-phosphate dehydrogenase A (GapA)
41	D-ribose-5-phosphate 3-epimerase (Rpe)
20	succinyl-CoA synthetase, alpha chain (SucD)

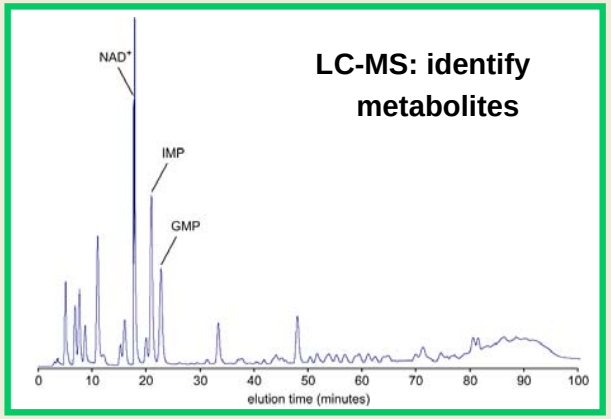
Protein gel: virulence factors expressed in flea vs human



Functional phenotype changes virulence factor expression



Why is expression so different due to carbon sources?



LC-MS: identify metabolites

Summary

- These studies have already found:
  - Indications of incomplete genome annotation
  - Changes in drug and osmotic susceptibility
  - Functional metabolic changes
  - Strain dependent phenotypes
- Linked virulence factor expression to carbon sources
- Opened new lines of inquiry for future research

LLNL Biodefense Proteomics Group

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Host Pathogen Interactions:
- Brett Chromy, Ph.D.
- Celia Zhang, Ph.D.
- Megan Choi
- Vicki Kopf
- Todd Corzett
- Michael Derksen
- Rachelle Bermingham

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- Cameron Forde, Ph.D. (UBC)

Statistician
- Imola Fodor, Ph.D.

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- Gilda Vanier

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- Brigitte Fisher
- Alexandra Robbins
- Kristin Robbins
- David Schroeder
- Josefina Seoane

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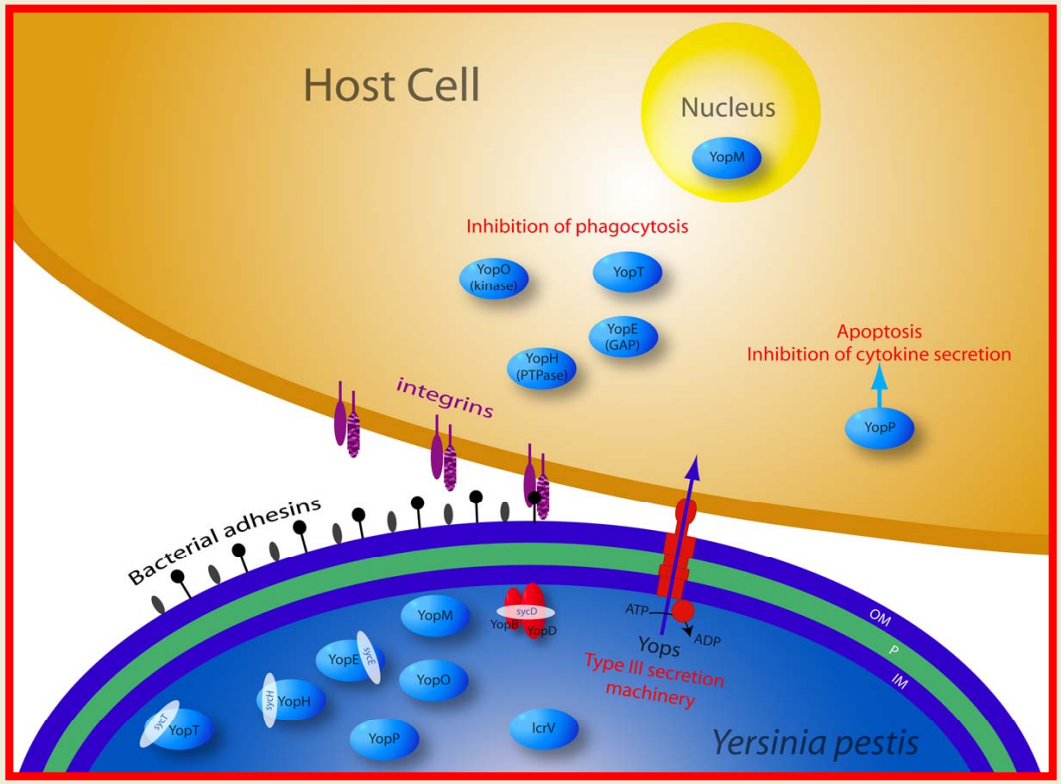
Collaborators

LLNL- P. Imbro, K. Smith, J.P. Fitch, M. Palmblad
Biocyc- Peter Karp, SRI International
Strains – E. Garcia, V. Motin, P. Imbro, Ft. Collins
UC Davis – J. Foley, N. Drazenovich
PNNL – M. Lipton, K. Hixon, D. Smith
Texas A&M – N. Clarke, G. Adams
Industry –Biolog

Host-pathogen Interaction Model (Infectomics)



Host

Cells (infection routes)
- Blood, Lung, Nasal

Whole Blood (human)

Animals

Human Studies

Pathogens

Y. Pestis

near neighbors: *Y. enterocolitica*, *Y. psuedotuberculosis*

B. anthracis

near neighbors: *B. cereus*, *B. thuringensis*

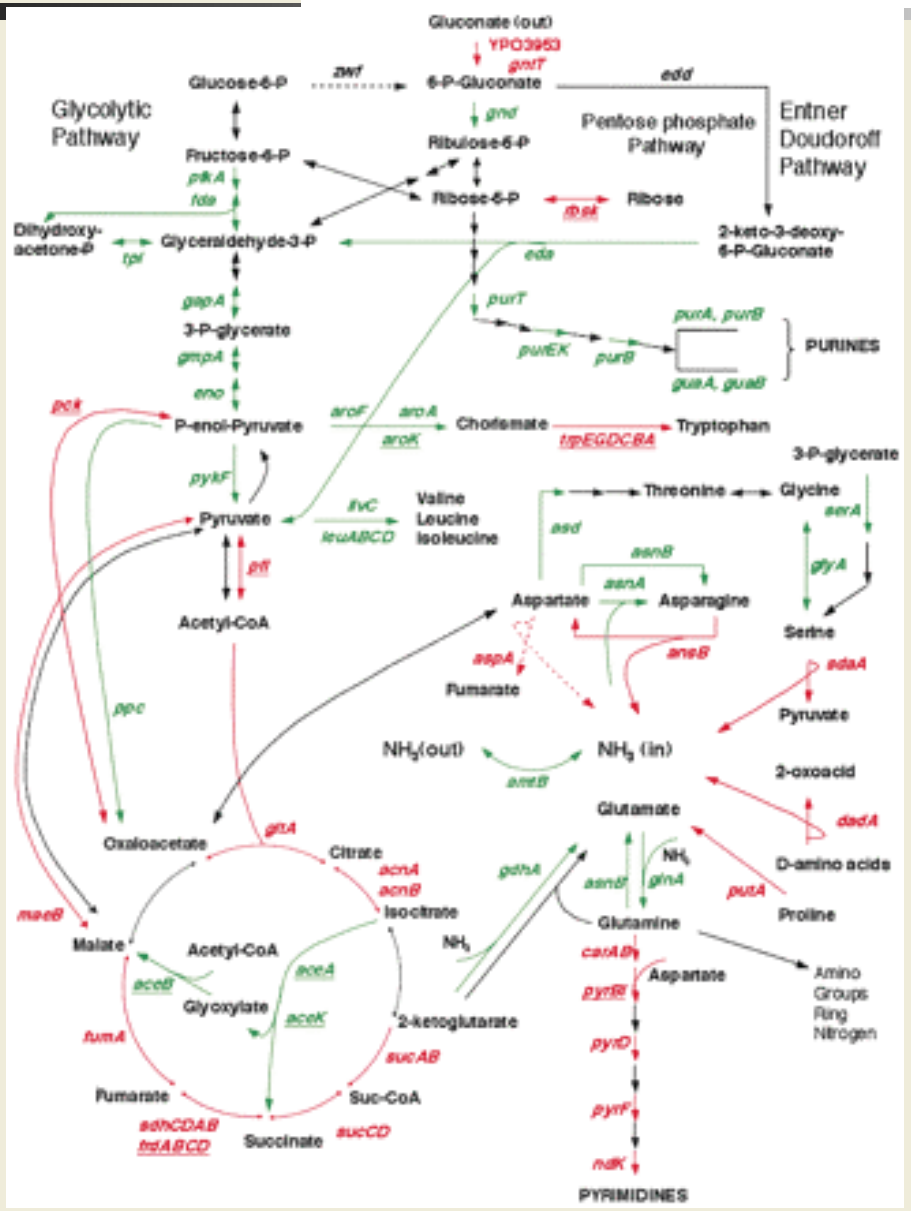
(Virulent v. avirulent)

Protein

2-D DIGE
Mass Spec.
Phenotype Arrays

→ Protein Biomarkers

Transcriptomics

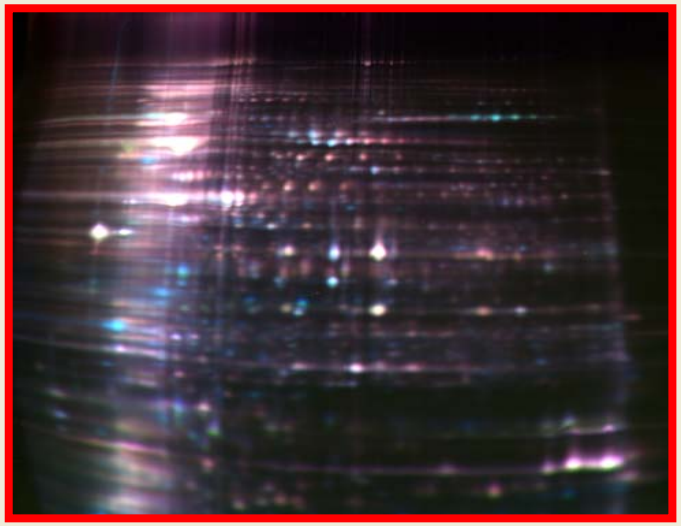


- Microarray data showed different expression levels
- Green are upregulated at 26°C-Ca
- Red are upregulated at 37°C-Ca
- Comparing RNA expression to protein expression to metabolic activity

Motin *et al.* 2004. J Bacteriol.

Yersinia pestis Proteomic Profiling

2-D DIGE



***Y. pestis* soluble Proteome**

26°C high calcium (flea)

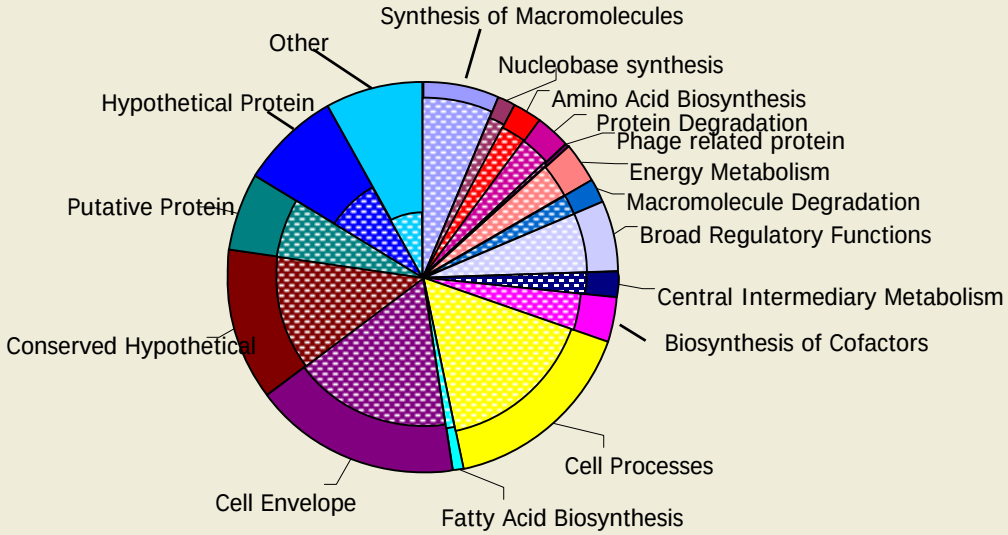
37°C low calcium (human)

>2800 proteins spots

Identified 239 differential proteins

Chromy, 2005 J Bacteriol.

FTICR-MS and accurate mass tags



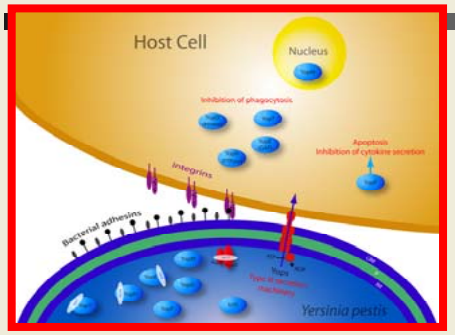
>5000 Proteins from Annotation

~35% proteome coverage

submitted

Collaboration with Drs. M. Lipton and D. Smith, PNNL, submitted

Host Response



Invited review 2005 *Exp Rev Prot*
Zhang et al. 2005 *Proteomics*
Chromy et al. 2004 *JPR*
Chromy et al. 2004 *BBRC*
Foley et al. submitted *Vet Med*

Host Models
Cells
Whole Blood
Animals
Human Clinical

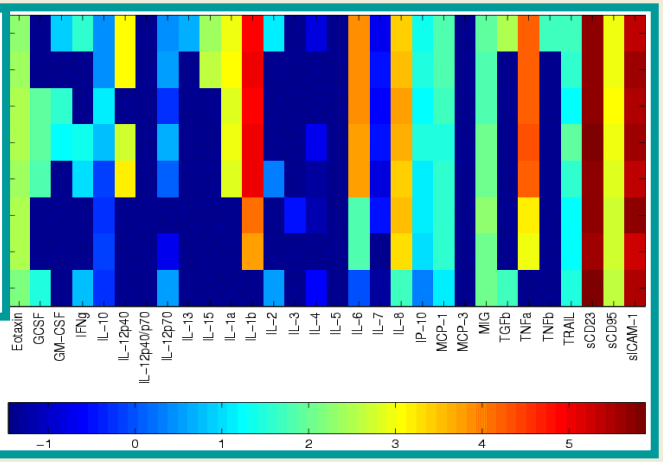
Pathogens
Y. pestis
B. anthracis
F. tularensis
Other strategic
Viruses, Toxins

Y.pe, Y.ps, Y.en
Distinct response

Cytokine Arrays

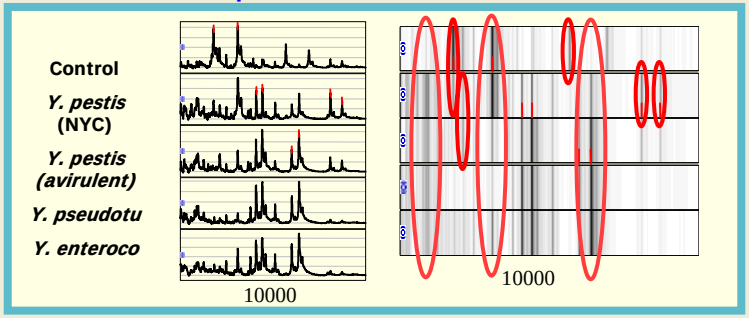
7 pathogens, plasma
Heat map of host response in human blood

Y. pestis **NYC**
Y. pestis **India**
Y. enterocolitica
Y. pseudotuberculosis
avirulent *Y. pestis*
B. anthracis
avirulent *B. anthracis*
Control

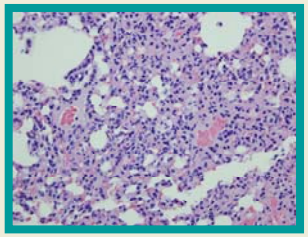


MS Screen

4 pathogen exposures, WBCs
IMAC-Cu chip

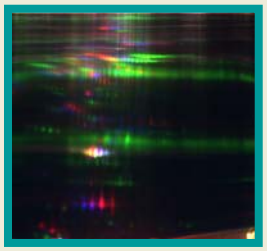


Animal Models



Rodent lung tissue
Collaboration Dr. J. Foley
Acute neutrophilic interstitial pneumonia w/ vascular reactivity

Lungs – lesions, *Y.pe* present
? pneumonic (alveoli) with NYC
RT PCR – Cytokine response
Deer mice – not resistant to NYC
CBCs – sepsis 3 days after exposure
Oxidative stress early
Spleen – severe necrosis
Lung, liver, spleen DIGE in progress



2-D DIGE Plasma
Monkey, Bovine
Human
Swine,
Rodent – in progress
(Plasma, Tissue, Saliva, Breath)

Future –> Dipstick, Mouthswab, Breathalyzer
Other Pathogens and Models –> bovine, swine, NHP

Technology

- Red dye accumulates as bacteria respire
- Omnilog robot (Biolog, Hayward CA) measures dye intensity in each well every 15 minutes
- Shown here, avirulent *Y. pestis* KIM D27 grown on antibiotics plate #15A with Calcium (top) and without Calcium (bottom)

Compare to “standard” phenotype in Biolog’s diagnostic database

Analyze mutants